APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2011, p. 4905–4911 Vol. 77, No. 14
0099-2240/11/$12.00 doi:10.1128/AEM.02872-10

Role Played by Exosporium Glycoproteins in the Surface Properties of *Bacillus cereus* Spores and in Their Adhesion to Stainless Steel▽

Yannick Lequette,[1] Estelle Garénaux,[4,5] Grégoire Tauveron,[1] Sylvain Dumez,[1] Stéphane Perchat,[2] Christian Slomianny,[3] Didier Lereclus,[2] Yann Guérardel,[4,5] and Christine Faille[1]*

INRA, UR638, F-59651 Villeneuve d'Ascq, France[1]; INRA, UMR1319, F-78285 Guyancourt Cedex, France[2]; INSERM, U1003, Université Lille 1, Cité Scientifique, Bât. SN3, F-59655 Villeneuve d'Ascq Cedex, France[3]; USTL, Unité de Glycobiologie Structurale et Fonctionnelle, UGSF, F-59650 Villeneuve d'Ascq Cedex, France[4]; and CNRS UMR 8576, F-59655 Villeneuve d'Ascq Cedex, France[5]

Received 8 December 2010/Accepted 9 May 2011

Bacillus cereus **spores are surrounded by a loose-fitting layer called the exosporium, whose distal part is mainly formed from glycoproteins. The role played by the exosporium glycoproteins of *B. cereus* ATCC 14579 (BclA and ExsH) was investigated by considering hydrophobicity and charge, as well as the properties of spore adhesion to stainless steel. The absence of BclA increased both the isoelectric point (IEP) and hydrophobicity of whole spores while simultaneously reducing the interaction between spores and stainless steel. However, neither the hydrophobicity nor the charge associated with BclA could explain the differences in the adhesion properties. Conversely, ExsH, another exosporium glycoprotein, did not play a significant role in spore surface properties. The monosaccharide analysis of *B. cereus* ATCC 14579 showed different glycosylation patterns on ExsH and BclA. Moreover, two specific glycosyl residues, namely, 2-*O*-methyl-rhamnose (2-Me-Rha) and 2,4-*O*-methyl-rhamnose (2,4-Me-Rha), were attached to BclA, in addition to the glycosyl residues already reported in *B. anthracis*.**

The food-borne pathogen *Bacillus cereus* has been extensively isolated in the form of spores from various environments, including food contact surfaces (26, 33). Spores of the *B. cereus* group (closely related species, such as *B. cereus*, *B. anthracis*, or *B. thuringiensis*) are characterized by the presence of an outer layer called the exosporium, which surrounds the spore and is suspected of playing a major role in the interface phenomena. Indeed, the exosporium has been shown to play a role in spore adhesion to abiotic surfaces (14) and to professional phagocytic cells (25), in the escape of spores from macrophages (27), and in spore germination (16). As revealed by electron microscopy, the exosporium is made of an external hair-like nap on top of a paracrystalline basal layer (18). The hair-like nap is mainly composed of the collagen-like glycoprotein BclA in *B. anthracis* (34). The BclA protein contains three domains: the N-terminal domain (NTD) of 44 amino acids (aa) is involved in targeting and anchoring the protein in the exosporium (38); the C-terminal domain (CTD) is composed of multiple β-strands (28); and the collagen-like region (CLR), with a triple-helix conformation (34), is composed of GXX repeats, mostly GPT, whose threonine residues provide putative glycosylation sites (8). The filament length is proportional to the number of GXX repeats (35). The presence of BclA at the surfaces of *B. anthracis* spores has been shown to have an impact on the overall hydrophobicity (6) and charge (7) of the spores. Furthermore, the presence of BclA affects the germination of *B. anthracis* spores and is involved in specific interaction with immune cells (4, 24) and surfactants (28). Genes of other collagen-like proteins are also present in the *B. anthracis* genome (22, 39), as well as in other *Bacillus* species, such as *exsH*, which encodes a protein with an NTD of 145 aa with putative helical secondary structures, or *exsJ* (40). However, their localization, composition, and role in spore properties have been poorly investigated, except for BclB (38).

The monosaccharide composition of BclA has so far been described only in *B. anthracis*. Two oligosaccharides have been characterized: a trisaccharide composed of 3-*O*-methyl-rhamnose (3-Me-Rha), L-rhamnose (Rha), and *N*-acetyl-galactosamine (GalNAc) residues and a pentasaccharide composed of one anthrose residue, three Rha residues, and a GalNAc residue (5, 8). Rha derivatives and GalNAc residues were also found in the BclB proteins of *B. anthracis* (42). While 3-Me-Rha, glucosamine ($GlcNH_2$), and galactosamine ($GalNH_2$) residues were found in the *B. cereus* exosporium (12), the reported results suggest significant differences in glycosylation patterns within *Bacillus* species and/or strains of *B. cereus*. First, 2-Me-Rha and 2,4-Me-Rha (12, 15), as well as fucose (15), absent from *B. anthracis* spores (8, 15), were identified in some *B. cereus* and *B. thuringiensis* strains, including *B. cereus* ATCC 14579. Elsewhere, anthrose was detected in *B. anthracis* and two *B. thuringiensis* spores but not in several *B. cereus* strains, including ATCC 14579 (8, 9). Lastly, Tamborrini et al. (36) showed that an antibody targeting the *B. anthracis* BclA tetrasaccharide reacted with spore extracts of some *B. cereus* strains, but not with *B. cereus* ATCC 14579. However, the glycosylation patterns of specific exosporium-associated glycoproteins have yet to be characterized in *B. cereus*.

The aim of this study was to investigate the influence of the BclA and ExsH glycoproteins on the ultrastructure of the *B. cereus* ATCC 14579 spore exosporium and on its physicochemical properties and adhesion to stainless steel.

* Corresponding author. Mailing address: INRA, UR638, F-59651 Villeneuve d'Ascq, France. Phone: 33 320435404. Fax: 33 320435426. E-mail: faille@lille.inra.fr.
▽ Published ahead of print on 27 May 2011.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
B. cereus		
ATCC 14579	Wild-type strain	
ATCC 14579 Δ*bclA*	Kan^r; *bclA*::*kan* deletion	This study
ATCC 14579 Δ*exsH*	Tet^r; *exsH*::*tet* deletion	This study
ATCC 14579 Δ*bclA* Δ*exsH*	Kan^r Tet^r; *bclA*::*kan* and *exsH*::*tet* deletions	This study
E. coli K-12		
TG1	Δ(*lac-proAB*) *supE thi hsd*Δ5 (F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)	
ET12567	F′ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 glaK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*	
Plasmids		
pHT304	Amp^r Ery^r; shuttle vector for *E. coli* and *B. cereus*	1
pRN5101	Thermosensitive vector	20
pDG786	Source of the Kan^r resistance cassette	17
pHTS2	Source of the Tet^r resistance cassette	30
pRN5101 Δ*bclA* ATCC 14579 Kan^r	Kan^r; plasmid used to delete *bclA*	This study
pRN5101 Δ*exsH* ATCC 14579 Tet^r	Tet^r; plasmid used to delete *exsH*	This study
pHT304 *bclA* ATCC 14579	Amp^r Ery^r; plasmids containing the *bclA* gene and 317 bp upstream and 138 bp downstream; used for complementation	This study
pHT304 *exsH* ATCC 14579	Amp^r Ery^r; plasmids containing the *exsH* gene and 465 bp upstream and 914 bp downstream; used for complementation	This study
pYL304	Amp^r Ery^{r}; plasmids expressing a BclA protein of 189 aa with the CTD deleted; *bclA*-ΔCT	This study
pYL306	Amp^r Ery^r; plasmid expressing a BclA protein of 45 aa with the CLR and CTD deleted; *bclA*-ΔCTCLR	This study

[a] Kan^r, kanamycin resistance; Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Ery^r, erythromycin resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Escherichia coli* strains and the *B. cereus* ATCC 14579 wild-type strain and its related mutants are listed in Table 1. Spores were produced as previously described (14). First, sporulation was carried out on plates for 10 days in order to obtain more than 95% mature spores. Then, the spores were scraped off the plates, washed 5 times with water, and stored at 4°C in order to kill the last few vegetative cells. The spores were used within 2 months. Before each experiment, two additional washing steps were carried out to remove most of the cell debris, and spore aggregates were disrupted by a sonication step (bath sonicator; 1 min 30 s twice at 42 kHz). All experiments were carried out on at least two independent spore batches. PCR using specific primers (Table 2) was performed on heated spores to verify the presence of the different alleles used in this study. The antibiotic resistance of the germinated spores was also checked for each batch. The *E. coli* K-12 TG1 strain was used for cloning experiments, and the *E. coli* ET12567 strain was used to generate unmethylated plasmid DNA prior to *B. cereus* electroporation. The following antibiotic concentrations were used when necessary: 100 μg/ml ampicillin and 20 μg/ml kanamycin for *E. coli* and 10 μg/ml tetracycline, 200 μg/ml kanamycin 116, and 10 μg/ml erythromycin for *B. cereus*.

Plasmids and mutant strains. The *bclA* and *exsH* genes of *B. cereus* ATCC 14579 were disrupted by inserting a kanamycin and a tetracycline resistance cassette, respectively. The thermosensitive plasmid pRN5101 was used to exchange the genes by homologous recombination as described previously (21). Briefly, the upstream and downstream regions of *bclA* and *exsH* were amplified using primers listed in Table 2. PCR fragments and a gene-resistant cassette (a Kan^r resistance cassette for *bclA* deletion and a Tet^r resistance cassette for *exsH* deletion) were cloned into the BamHI-HindIII site of pRN5101 (Table 1). Unmethylated plasmids were electroporated into the *B. cereus* ATCC 14579 strain, and Kan^r or Tet^r resistant clones were selected for the deletion of *bclA* and *exsH*, respectively. Double recombinants were selected by screening for Kan^r and Tet^r clones sensitive to erythromycin. The double recombinant was verified by PCR. The resulting strains were designated ATCC 14579 Δ*bclA* and ATCC 14579 Δ*exsH*, respectively. The double-mutant strain ATCC 14579 Δ*bclA* Δ*exsH* was constructed by electroporating the ATCC 14579 Δ*bclA* strain with pRN5101 Δ*exsH* ATCC 14579 Tet^r. To perform the complementation, a 1.2-kb EcoRI-HindIII fragment containing the *bclA* gene and its own promoter was amplified by PCR and cloned into the digested EcoRI-HindIII pHT304 vector. pHT304 is a shuttle vector (*E. coli-B. cereus*) with a low copy number in *B. cereus* (~4 copies

TABLE 2. Primers used in this study

Primer	Sequence	Purpose
BclI	5′-CCCAAGCTTTCATAGCAATCTCCTAAC-3′	For amplification upstream of *bclA*
Bcll2	5′-AACTGCAGTAGATGCAAAACCGAAAGAAAA-3′	
Bcl3	5′-AACGAGCTCGTTACAGGACTTGGGCTATCA-3′	For amplification downstream of *bclA*
Bcl4	5′-CGGGATCCAT TGTGGATTCGTACATATC-3′	
exsH1bis	5′-CGGGATCCTATACATTTCCGATTCT-3′	For amplification upstream of *exsH*
exsH4	5′-GGAATTCACACATGAAGCTTGGACCCCTTTGTATTATAG-3′	
exsH71	5′-GCTCTAGAGCTGCAGTTGCTGGATTTGTAAGT-3′	For amplification downstream of *exsH*
exsH8bis	5′-CCCAAGCTTTAAAACCCGGGCAAGTTGC-3′	
Bcl5	5′-CCGGAATTCTAAACAACGGGCTATTGTCTC-3′	For amplification of the *bclA* gene
Bcl6	5′-CCCAAGCTTTCCATATTTGTGCCTCCTGC-3′	
exsI7	5′-GCTCTAGAATAAATGGTTGAATGATAGGAAT-3′	For amplification of the *exsH* gene
exsH20	5′-TAATGACGTAATAACCCCTGCTCT-3′	
exsH19	5′-GCTCTTGCACCATTCTTCTTCTC-3′	

per equivalent chromosome) (1) (Table 1). The entire *exsH* gene with its own promoter was amplified in two steps. First, two overlapping fragments were generated using the exsI7-exsH20 and exsH19-exsH8bis primers (Table 2). The two fragments were used as primers to each other in a second PCR in order to generate the *exsH* gene, which was cloned into the digested XbaI-HindIII pHT304 vector (Table 1).

Characterization of the glycoprotein oligosaccharide moieties. For the determination of the monosaccharide composition of exosporium fractions, spores were washed twice in water, resuspended in water, and subjected to four successive passages through a French press at 20,000 lb/in^2. After elimination of spores by centrifugation (3,000 × *g*; 30 min; 4°C), insoluble fractions of exosporia were pelleted by ultracentrifugation (120,000 × *g*; 30 min; 4°C). The monosaccharide composition of the exosporium fraction was established by gas chromatography (GC) and GC-mass spectrometry (MS) as alditol-acetate derivatives. Briefly, samples were hydrolyzed in 4 M trifluoroacetic acid (TFA) for 4 h at 100°C and then reduced with sodium borohydride in 0.05 M NaOH for 4 h. Reduction was stopped by dropwise addition of acetic acid until pH 6 was reached, and borate salts were codistilled by repetitive evaporation in dry methanol. Peracetylation was performed in acetic anhydride at 100°C for 2 h. A galactosyl residue (Gal) was always detected in *B. cereus* exosporium samples, but the large quantitative batch-to-batch variability strongly suggested it originated, at least partly, from ubiquitous agarose contamination, and it was therefore excluded from further glycosyl residue dosages.

Transmission electron microscopy (TEM). Whole spores were adsorbed onto Formvar-coated grids (Formvar 15/95; Euromedex, EMS, Hatfield, PA) and examined after negative staining with 1% uranyl acetate (VWR, Fontenay-sous-Bois, France) on a Hitachi H600 electron microscope at a 75-kV accelerated voltage (12). In order to allow the observation of nanofeatures, ultrathin sections were also observed by TEM after sectioning. Since the external part of exosporia is mainly composed of glycoproteins, a ruthenium red staining procedure previously described (41), and slightly modified in our laboratory, was performed (14). The lengths of filaments were measured using ImageJ software.

MATH. A kinetic microbial affinity to hydrocarbon (MATH) method previously described (2) was slightly modified. Hexadecane (Sigma) was chosen as an apolar solvent. Briefly, spores were resuspended in 3 ml of NaCl (0.9% [wt/vol]) at an absorbance of 0.6 at 600 nm in glass tubes. Hexadecane (500 μl) was added to the spore suspension, and the glass tubes were vortexed for different times ranging from 5 to 150 s. Then, the glass tubes were left to settle for 30 min to reach complete separation of the two phases. The absorbance of the aqueous phase was measured at 600 nm (A_t). Log [(A_t/A_0) × 100] was plotted against the vortexing time. The initial slope represents the initial removal rate (R_0 [min^{-1}]) of spores from the aqueous suspension and is related to the hydrophilic/hydrophobic spore character.

Measurements of spore electrical properties. The electrical properties of spores were measured by microelectrophoresis using a Zeta Compact zetameter (CAD Instruments, Les Essarts-le-Roi, France). The electrophoretic motilities were determined using the Helmotz-Smoluchowski equation. Solutions at pH 2.8, 3, 4, 5, 6, 7, 8, and 9 were obtained by combining KOH, KNO_3, and HNO_3 solutions (1 mM). Spores were suspended in each solution (50 ml) to obtain 40 to 60 spores per reading.

Detachment of spores from stainless steel slides using normal stress. Spores were suspended (10^3 to 10^5 spores/ml) in 300 ml of distilled water (the exact concentration of spores was determined by plating a serial dilution of the suspension on nutrient agar plates). Stainless steel slides (304L; bright annealed; 70 mm by 65 mm) were vertically immersed in the spore suspension for 4 h, allowing spores to adhere. The slides were then rinsed five times in water to eliminate loosely attached spores and dried at 50°C for 45 min. Using an ATL plate applicator, 10 contact agar plates (nutrient agar plus triphenyl tetrazoliumchloride [TTC]; ATL) were successively applied to each stainless steel slide for 10 s at a force of 2,451 Pa (according to ATL's instructions) and lifted off to remove spores. After the 10 contacts, the slide was covered with a solid layer of TTC-agar (2 to 3 mm wide). The contact agar plates and stainless steel slides covered with TTC-agar were incubated at 30°C for 48 h. The colonies formed on each contact agar plate corresponded to the number of spores (N_i) removed at the contact (i). The number of spores remaining on the slide after the 10 contacts (N_r) was determined by the number of colonies developed in the TTC-agar layer covering the stainless steel slides. The equation $f(i) = \{[N_0 - \sum(N_i)]/N_0\} \times 100$ was plotted. The initial number of adherent spores, N_0, was calculated as the sum of the colonies on each contact agar plate plus N_r. The number of contacts required to remove 50% of the spores was calculated in order to compare the adhesion properties of mutant spores. Experiments were repeated at least 3 times with two different batches of spores.

Nucleotide sequence accession number. The new sequence of the *bclA* gene of the *B. cereus* ATCC 14579 strain is available in the NCBI database under accession no. HM071986.1.

RESULTS AND DISCUSSION

Characterization of the exosporium glycoproteins of *B. cereus* ATCC 14579. The *bclA* open reading frame (ORF) (*bc1207*; GenBank accession number AAPO8192.1) annotated in the *B. cereus* ATCC 14579 genome of the NCBI database predicts a 128-aa protein with very short CLR and CTD compared to the BclA of *B. anthracis*. Our cloning and sequencing of the *bclA* gene of the *B. cereus* ATCC 14579 strain showed an 888-bp-long ORF. A 295-amino-acid protein (27.7 kDa) was predicted to have an NTD identical to that of *B. anthracis* Sterne BclA, a CLR containing 41 GXX collagen-like motifs (shorter than in *B. anthracis* BclA), and a CTD with 89% identity to that of *B. anthracis* Sterne BclA.

A *B. cereus* ATCC 14579 strain with the *bclA* gene deleted was created to characterize the role of BclA in the exosporium integrity and spore surface properties. Ultrathin sections of wild-type spores observed by TEM showed a dense and regular hair-like nap with 36- ± 1-nm-long filaments (Fig. 1A). Conversely, spores with *bclA* deleted exhibited only a little material, which was unevenly distributed at the exosporium surface (Fig. 1B), but apart from the lack of the well-structured hair-like nap, the exosporium did not seem to be affected by the *bclA* deletion.

The complementation of the *bclA* mutation restored a hair-like nap similar to that of the wild-type strain (Fig. 1C). The material around the Δ*bclA* spores was observed whatever the spore batch and could be another glycoprotein component of the nap or fragments of the inner part of the exosporium, which would slough off in the absence of BclA. It could also be supposed that the material comes from the vegetative cell or even the growth medium and adhered to the spore surface due to unusual adhesive properties of the spore surface revealed following *bclA* deletion. Elsewhere, the presence of such material unevenly distributed at the exosporium surface has not previously been reported on *B. anthracis* spores with *bclA* deleted (3, 34). In order to check if the presence of this material was due to the experimental conditions used in this study, we performed similar observations on the avirulent strain *B. anthracis* 9131 (10), lacking both the pXO1 and pXO2 plasmids, and its mutant with *bclA* deleted (35), both provided by the Institut Pasteur (Paris, France) (Fig. 1G and H, respectively). Observation by TEM revealed no material around the spores, thus suggesting quite different compositions of the external layers of *B. cereus* ATCC 14579 and *B. anthracis* 9131 spores.

The *B. cereus* ATCC 14579 genome contains other genes encoding BclA-like glycoproteins. One of them, *exsH*, encodes a protein with a 145-aa NTD containing putative helical secondary structures absent from the 44-aa NTD of BclA. We therefore investigated the role of this protein in the exosporium properties by deleting the *exsH* ORF. The deletion of *exsH* did not cause any visible alterations to the exosporium, which remained intact and surrounded with a hair-like nap (Fig. 1D) similar to that observed around the wild-type spores. This observation suggests that ExsH does not play a major role in the exosporium structure and that BclA is the major protein

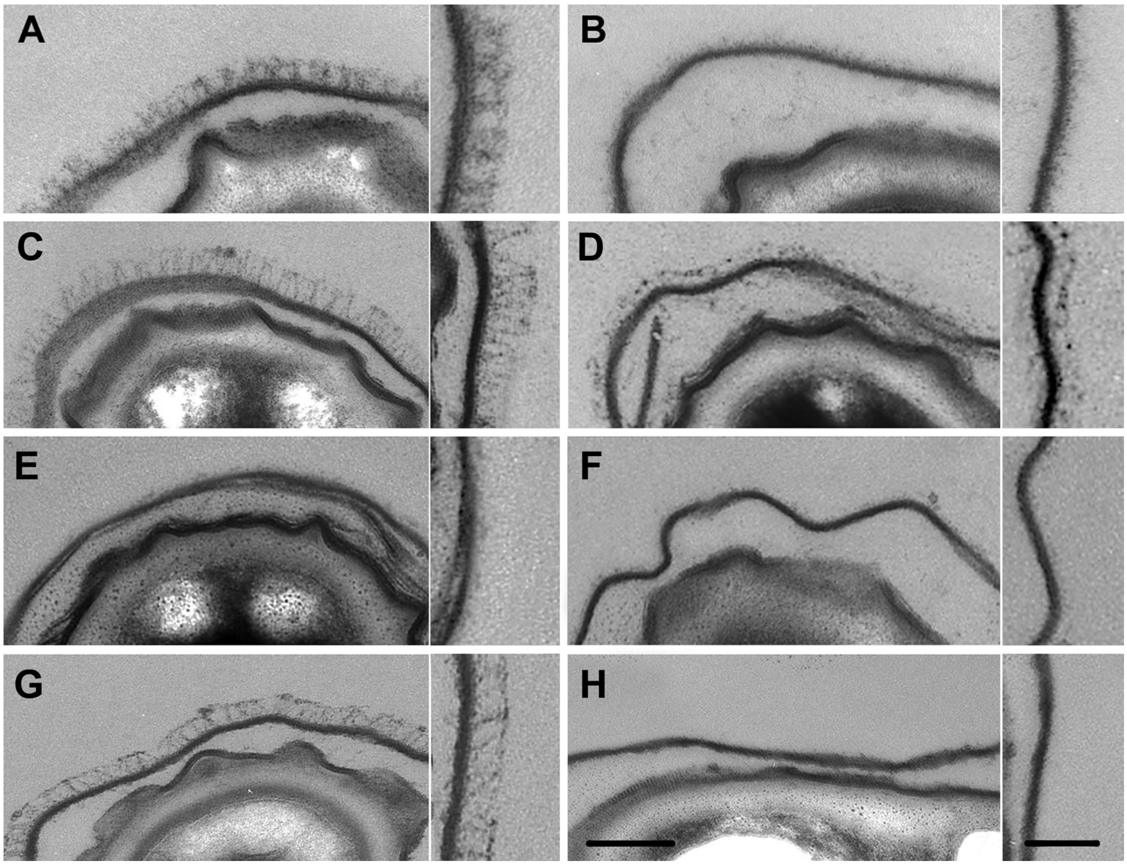

FIG. 1. Ultrathin sections of *B. cereus* ATCC 14579 and *B. anthracis* 9131 spores and the related mutants visualized by transmitted electron microscopy (scale bar = 100 nm). For each strain, the inset is an enlargement of a representative part of the corresponding exosporium (scale bar = 50 nm). (A to F) *B. cereus* ATCC 14579. (A) Wild type. (B) Δ*bclA*. (C) Δ*bclA* pHT304 *bclA*. (D) Δ*exsH*. (E) Δ*bclA* Δ*exsH*. (F) Δ*bclA* Δ*exsH* pHT304 *exsH*. (G and H) *B. anthracis* 9131. (G) Wild type. (H) Δ*bclA*.

of the hair-like nap, as in *B. anthracis* (34). In order to verify that BclA did not mask any minor modifications to the exosporium structure following *exsH* deletion, the ATCC 14579 Δ*bclA* Δ*exsH* double mutant was created. The *exsH* gene under the control of its own promoter was expressed on a multicopy plasmid in the double mutant. Its expression was confirmed by analysis of the glycosyl moieties of the different mutants. Some material was still observed by TEM at the spore surface of the double mutant (Fig. 1E) and of the double mutant complemented with *exsH* (Fig. 1F). There was no significant difference in the material at the exosporium surfaces of the three mutants with *bclA* deleted. This result indicates that the material was not made of ExsH but did not permit us to draw definitive conclusions about the origin of the material (spore, vegetative cell, or growth medium).

Lastly, the presence of the long appendages, about 10 nm wide (12, 13), was not affected by either *bclA* or *exsH* deletion (data not shown).

Identification of the monosaccharide composition of BclA and ExsH glycoproteins. As shown recently (12), along with Rha, $GalNH_2$, and $GlcNH_2$, the major rhamnose derivatives found in the exosporium of *B. cereus* ATCC 14579 were 2-Me-Rha, 3-Me-Rha, and 2,4-diMe-Rha residues. Rha, $GalNH_2$, $GlcNH_2$, and 3-Me-Rha have been previously identified in *B. anthracis* (8), while 2-Me-Rha and 2,4-diMe-Rha have been described in a few *B. cereus* and *B. thuringiensis* strains (12). The method used here to analyze monosaccharide composition did not permit us to discriminate NH_2 from *N*-acetyl groups and, thus, $GalNH_2$-$GlcNH_2$ from GalNAc-GlcNAc. The identification of GalNAc, rather than a $GalNH_2$ residue, in *B. anthracis* spores strongly suggested that the $GalNH_2$ and $GlcNH_2$ residues identified in *B. cereus* strains also derived from *N*-acetyl-galactosamine and *N*-acetyl-glucosamine (8). The monosaccharide compositions of *bclA* and *exsH* mutants were analyzed to investigate their relative contributions to the global glycosylation of the *B. cereus* exosporium. The quantities of all Rha derivatives and $GalNH_2$ and $GlcNH_2$ residues were below the detection level in exosporia of strains with *bclA* alone deleted and with both *bclA* and *exsH* genes deleted (Fig. 2). In comparison, the deletion of *exsH* had no significant influence on the overall monosaccharide content of the exosporium. Complementation of the *bclA* deletion restored a profile similar to that of the wild type. These results established that the exosporium glycosylation was mainly BclA borne, irrespective of the monosaccharide residue considered. In order to identify the monosaccharide composition of ExsH, otherwise masked by the dominant glycosylation moieties associated with BclA, the *exsH* gene was expressed on a multicopy plasmid in the *B.*

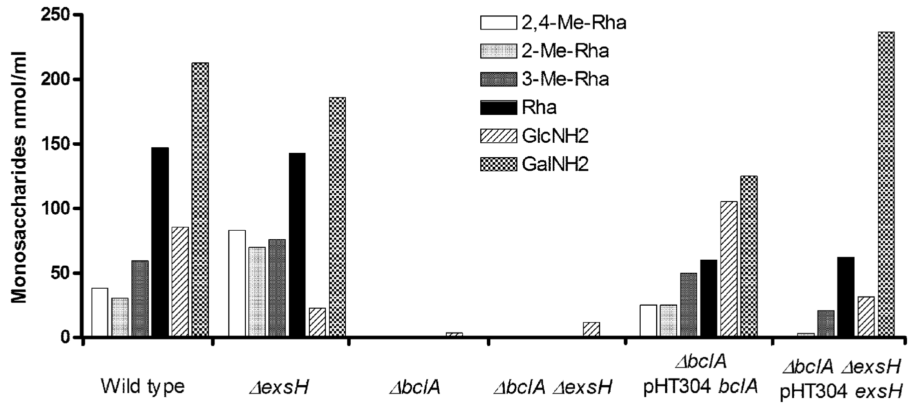

FIG. 2. Monosaccharide contents of exosporium fractions. The main monosaccharides in the *B. cereus* exosporium were identified and quantified as alditol-acetates in gas chromatography from the wild-type *B. cereus* ATCC 14579 and the related mutants with the *bclA* gene and the *exsH* gene deleted. The Δ*bclA* and the double Δ*bclA* Δ*exsH* mutants were complemented with plasmids pHT304 *bclA* ATCC 14579 and pTH304 *exsH* ATCC 14579, respectively.

cereus ATCC 14579 Δ*bclA* Δ*exsH* double mutant. $GalNH_2$, $GlcNH_2$, Rha, and 3-Me-Rha residues were detected, but 2-Me-Rha and 2,4-Me-Rha residues were not (Fig. 3). These results suggested that the ExsH protein was indeed glycosylated and exhibited a glycosylation pattern different from that of *B. cereus* ATCC 14579 BclA but similar to that of *B. anthracis* BclA (8). This result also confirmed that *exsH* was expressed from the multicopy plasmid in the *bcla exsH* mutant background but must be a minor protein in the exosporium, not observed by TEM. It is noteworthy that the Rha derivatives in *B. anthracis* are involved in specific recognition of the macrophage receptor CD14 (5, 24). The presence of several surface glycoproteins differently glycosylated on the *B. cereus* ATCC 14579 exosporium suggests specific recognition of different cell receptors. In particular, the specific glycosyl residues on BclA of *B. cereus* ATCC 14579 might mediate the recognition of host cell receptors different from the ones recognized by *B. anthracis*.

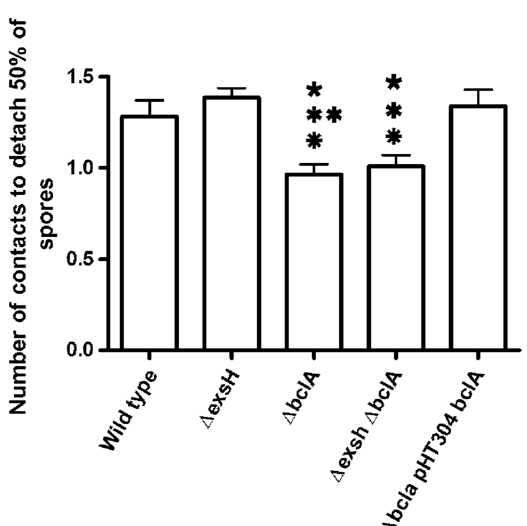

FIG. 3. Comparison of properties of spore adhesion to stainless steel. The numbers of contacts required to remove 50% of spores from stainless steel slides (304L, bright annealed) are represented by bars. One-way analysis of variance (ANOVA) was performed using Bonferroni's multiple-comparison test. The five-pointed asterisk represents a significant difference between a mutant and the ATCC 14579 wild type, the six-pointed asterisk represents a significant difference between a mutant and ATCC 14579 Δ*exsH*, and the eight-pointed asterisk represents a significant difference between a mutant and ATCC 14579 Δ*bclA* pHT304 *bclA*. One symbol represents a P value of <0.05 and two symbols a P value of <0.01. The error bars indicate standard deviations.

Influence of BclA and ExsH glycoproteins on both hydrophobic and electrical properties of the whole spores. Due to their external localization, BclA and ExsH proteins are good candidates for modulating spore surface properties. Thus, the hydrophobic character of *bclA* and *exsH* mutant spores was estimated from the initial removal rate (R_0) measured with the MATH assay (2, 23). The R_0 of wild-type *B. cereus* ATCC 14579 spores was measured at -4.39 ± 0.16 s^{-1}, indicating that wild-type spores were mostly hydrophobic. The R_0 of Δ*bclA* spores was even more negative than that of the wild type, revealing an increase in the spore hydrophobicity (Table 3). The complementation of the *bclA* mutation only partially restored the initial hydrophobicity, and the difference could not be attributed to the presence of the pHT304 vector. Indeed, the insertion of the empty pHT304 vector in the wild-type strain did not modify spore hydrophobicity (data not shown). Lastly, the deletion of *exsH* in the wild type or in the *bclA* mutant did not cause any significant change in spore hydrophobicity compared to the parental strains (Table 3).

Despite the increased hydrophobicity of the whole spores resulting from the *bclA* deletion, the GRAVY score (0.100) calculated from the BclA sequence predicted a hydrophobic character (20). This result suggested that the overall hydrophobicity of *B. cereus* spores is driven by underlying hydrophobic proteins and/or lipids (2) rather than by the external BclA. The opposite results have been reported for *B. anthracis* spore hydrophobicity following *bclA* deletion (7) in spite of the similar methods used in the two studies to estimate the spore hydrophobicity (affinity to hexadecane and brief contact). One may hypothesize that the underlying layers of the two species are quite different, at least in their hydrophobic characters.

The influence of *bclA* and *exsH* mutations on the electrical

TABLE 3. Physicochemical properties of spores[a]

Strain	Hydrophobicity			IEP		
	R_0 (min^{-1})	SD	P	Value	SD	P
ATCC 14579	−4.39	0.16		3.05	0.05	
ATCC 14579 Δ*bclA*	−6.33	0.43	<0.001	3.35	0.05	<0.01
ATCC 14579 Δ*exsH*	−4.47	0.28	ND	3.06	0.08	ND
ATCC 14579 Δ*bclA* Δ*exsH*	−6.32	0.08	<0.001	3.56	0.05	<0.001
ATCC 14579 Δ*bclA* pHT304 *bclA*	−5.16	0.14	ND	3.10	0.06	ND
ATCC 14579 Δ*bclA* pHT304 Δ*CT-bclA*	−7.01	0.18	<0.001	3.42	0.04	<0.05
ATCC 14579 Δ*bclA* pHT304 Δ*CT-CLR-bclA*	−6.40	0.14	<0.05	3.62	0.07	<0.001

[a] The hydrophobicity was estimated by least-square fitting, yielding a negative initial removal rate (R_0 [min^{-1}]). Statistical analyses comparing the wild type to each mutant were performed using Bonferroni's multiple-comparison test. ND, not statistically different from the control. SD, standard deviation.

properties of spores was measured using a zetameter. The isoelectric point (IEP) was used to compare strains. The IEP of spores statistically increased when the *bclA* gene was deleted (Table 3). The complementation of the *bclA* mutation restored the IEP, similar to the wild type. In comparison, the deletion of *exsH* in the wild type or in the *bclA* mutant did not significantly change the IEP compared to their parental strains. These results showed that BclA, but not ExsH, affected the overall charge of *B. cereus* spores. The variation in the BclA sequence and glycan moieties observed between species of the *B. cereus* group may influence the overall charge of spores.

Influences of BclA and ExsH glycoproteins on adhesion properties of *B. cereus* ATCC 14579 spores to stainless steel. We then investigated the influence of *bclA* and *exsH* deletions on the properties of *B. cereus* ATCC 14579 spore adhesion to stainless steel slides. The results showed that the absence of BclA in the hair-like nap significantly reduced ($P < 0.05$) adherent spores' resistance to detachment (Fig. 3). The complementation of the *bclA* mutation restored the adhesion properties to a level similar to that of the wild-type spores. Therefore, BclA increased the interaction force between spores and stainless steel. The deletion of the *exsH* gene did not cause any significant changes in the adhesion properties of spores compared to the parental strains.

We then investigated if the reduced resistance to detachment could be attributed to differences in spore surface physicochemistry (increased hydrophobicity and IEP). Numerous works have reported the role of hydrophobicity in spore interaction with materials. However, although it is generally admitted that spore hydrophobicity promotes spore adhesion to inert hydrophobic surfaces (11, 12, 29, 32), published studies failed to demonstrate any influence of spore hydrophobicity on their resistance to detachment (37). Furthermore, recent results obtained in our laboratory on 16 *Bacillus* strains from 7 *Bacillus* species, characterized by different surface hydrophobicities, showed that the mechanical detachment of adherent spores was not affected by their surface hydrophobicity (data not shown). We then investigated the role of electrostatic charge on the removal of adherent spores by comparing spore behavior at pH 7.0 and at pH 3.0. Indeed, spores of *B. cereus* ATCC 14579 and its mutants are negatively charged at pH 7.0, and their IEP is close to pH 3.0. As most materials are also negatively charged at neutral pH, the electrostatic repulsion occurring at pH 7.0 would limit spore adhesion. Indeed, previous studies have shown that a higher number of *Bacillus* spores adhere to stainless steel at pH 3.0 than at pH 7.0 (19, 31). Conversely, we showed that the resistance to detachment of adherent spores of both strains (the wild type and the Δ*bclA* strain) increased by only about 1.25-fold at pH 3.0 compared to pH 7.0. This result suggested that the spore charge poorly affected spore resistance to detachment. Therefore, the BclA-borne charges exhibit a marginal influence on the electrostatic repulsion of spores by stainless steel.

In conclusion, the study showed that BclA was the major glycoprotein of the exosporium hair-like nap of *B. cereus* strain ATCC 14579. The removal of BclA affected both the overall hydrophobicity and charge of spores, as well as their adhesion properties, but it did not affect exosporium integrity. The presence of BclA filaments of the hair-like nap plays a major role in spore interaction with materials, contrary to overall spore physicochemistry, probably by providing a larger contact surface with stainless steel. The monosaccharide composition of glycoproteins in the exosporium of *B. cereus* ATCC 14579 was different from that of the *B. anthracis* exosporium. Variations in the glycan moieties inside the *B. cereus* group may be responsible for different specificities of binding to host cells. The identification of the critical domains and amino acids of BclA involved in spore adhesion would help in the development of new compounds and processes to remove *B. cereus* spores from surfaces and host cells. Minor glycoproteins, such as BclB in *B. anthracis* and ExsH in *B. cereus*, as well as the multitude of putative glycoprotein genes present in the *B. cereus* group genomes (22, 40), could be involved in the differentiation of ligands at the surfaces of host cells. Conversely, we showed in this study that the ExsH protein is not involved in exosporium integrity, in the overall surface properties of spores, or in their adhesion properties.

ACKNOWLEDGMENTS

We thank Martine Clarisse, Etienne Dewailly, Véronique Lebret, Sylviane Parent, and Annette Ronse for their valuable assistance.

This work has been financed by the Agence Nationale de la Recherche under the Programme National de Recherche en Alimentation et Nutrition Humaine, project ANR-05-PNRA-013, *B. cereus*, and project ANR-07-PNRA-009, InterSpore.

REFERENCES

1. **Arantes, O., and D. Lereclus.** 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene **108:**115–119.
2. **Bos, R., and H. J. Busscher.** 1999. Role of acid-base interactions on the adhesion of oral streptococci and actinomyces to hexadecane and chloroform. Influence of divalent cations and comparison between free energies of partitioning and free energies obtained by extended DLVO analysis. Coll. Surf. B Biointerfaces **14:**169–177.
3. **Boydston, J. A., P. Chen, C. T. Steichen, and C. L. Turnbough.** 2005. Ori-

entation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. J. Bacteriol. **187:**5310–5317.

4. **Bozue, J., et al.** 2007. *Bacillus anthracis* spores of the *bclA* mutant exhibit increased adherence to epithelial cells, fibroblasts, and endothelial cells but not to macrophages. Infect. Immun. **75:**4498–4505.
5. **Bozue, J. A., et al.** 2005. Construction of a rhamnose mutation in *Bacillus anthracis* affects adherence to macrophages but not virulence in guinea pigs. Microb. Pathog. **38:**1–12.
6. **Brahmbhatt, T. N., et al.** 2007. *Bacillus anthracis* exosporium protein Bc1A affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. Infect. Immun. **75:**5233–5239.
7. **Chen, G., A. Driks, K. Tawfiq, M. Mallozzi, and S. Patil.** 2010. *Bacillus anthracis* and *Bacillus subtilis* spore surface properties and transport. Coll. Surf. B Biointerfaces **76:**512–518.
8. **Daubenspeck, J. M., et al.** 2004. Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. J. Biol. Chem. **279:**30945–30953.
9. **Dong, S. L., et al.** 2008. Anthrose biosynthetic operon of *Bacillus anthracis*. J. Bacteriol. **190:**2350–2359.
10. **Etiennetoumelin, I., J. C. Sirard, E. Duflot, M. Mock, and A. Fouet.** 1995. Characterization of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. J. Bacteriol. **177:**614–620.
11. **Faille, C., et al.** 2002. Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. Can. J. Microbiol. **48:**728–738.
12. **Faille, C., et al.** 2010. Morphology and physico-chemical properties of *Bacillus* spores surrounded or not with an exosporium: consequences on their ability to adhere to stainless steel. Int. J. Food Microbiol. **143:**125–135.
13. **Faille, C., et al.** 2010. Viability and surface properties of spores subjected to a cleaning-in-place procedure. Consequences on their ability to contaminate surfaces of equipment. Food Microbiol. **27:**769–776.
14. **Faille, C., G. Tauveron, C. Le Gentil-Lelièvre, and C. Slomianny.** 2007. Occurrence of *Bacillus cereus* spores with a damaged exosporium: Consequences on the spore adhesion on surfaces of food processing lines. J. Food Prot. **70:**2346–2353.
15. **Fox, A., et al.** 2003. Carbohydrates and glycoproteins of *Bacillus anthracis* and related bacilli: targets for biodetection. J. Microbiol. Methods **54:**143–152.
16. **Giorno, R., et al.** 2007. Morphogenesis of the *Bacillus anthracis* spore. J. Bacteriol. **189:**691–705.
17. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
18. **Henriques, A. O., and C. P. Moran.** 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. **61:**555–588.
19. **Hüsmark, U., and U. Rönner.** 1992. The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of *Bacillus* spores. Biofouling **5:**335–344.
20. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
21. **Lereclus, D., M. Vallade, J. Chaufaux, O. Arantes, and S. Rambaud.** 1992. Expansion of insecticidal host range of *Bacillus thuringiensis* by *in vivo* genetic recombination. Biotechnology **10:**418–421.
22. **Leski, T. A., et al.** 2009. Identification and classification of *bcl* genes and proteins of *Bacillus cereus* group organisms and their application in *Bacillus anthracis* detection and fingerprinting. Appl. Environ. Microbiol. **75:**7163–7172.
23. **Lichtenberg, D., M. Rosenberg, N. Sharfman, and I. Ofek.** 1985. A kinetic approach to bacterial adherence to hydrocarbon. J. Microbiol. Methods **4:**141–146.
24. **Oliva, C., C. L. Turnbough, and J. F. Kearney.** 2009. CD14-Mac-1 interactions in Bacillus anthracis spore internalization by macrophages. Proc. Natl. Acad. Sci. U. S. A. **106:**13957–13962.
25. **Oliva, C. R., et al.** 2008. The integrin Mac-1 (CR3) mediates internalization and directs *Bacillus anthracis* spores into professional phagocytes. Proc. Natl. Acad. Sci. U. S. A. **105:**1261–1266.
26. **Rajkovic, A., M. Uyttendaele, T. Courtens, M. Heyndrickx, and J. Debevere.** 2006. Prevalence and characterisation of *Bacillus cereus* in vacuum packed potato puree. Int. J. Food Sci. Technol. **41:**878–884.
27. **Ramarao, N., and D. Lereclus.** 2005. The InhA1 metalloprotease allows spores of the *B. cereus* group to escape macrophages. Cell Microbiol. **7:**1357–1364.
28. **Rety, S., et al.** 2005. The crystal structure of the *Bacillus anthracis* spore surface protein BclA shows remarkable similarity to mammalian proteins. J. Biol. Chem. **280:**43073–43078.
29. **Rönner, U., U. Hüsmark, and A. Henriksson.** 1990. Adhesion of *Bacillus* spores in relation to hydrophobicity. J. Appl. Bacteriol. **69:**550–556.
30. **Sanchis, V., H. Agaisse, J. Chaufaux, and D. Lereclus.** 1996. Construction of new insecticidal *Bacillus thuringiensis* recombinant strains by using the sporulation non-dependent expression system of cryIIIA and a site specific recombination vector. J. Biotechnol. **48:**81–96.
31. **Seale, R. B., P. J. Bremer, S. H. Flint, and A. J. McQuillan.** 2010. Characterization of spore surfaces from a Geobacillus sp. isolate by pH dependence of surface charge and infrared spectra. J. Appl. Microbiol. **109:**1339–1348.
32. **Simmonds, P., B. L. Mossel, T. Intaraphan, and H. C. Deeth.** 2003. Heat resistance of *Bacillus* spores when adhered to stainless steel and its relationship to spore hydrophobicity. J. Food Prot. **66:**2070–2075.
33. **Svensson, B., et al.** 2006. Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. Int. Dairy J. **16:**740–749.
34. **Sylvestre, P., E. Couture-Tosi, and M. Mock.** 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. Mol. Microbiol. **45:**169–178.
35. **Sylvestre, P., E. Couture-Tosi, and M. Mock.** 2003. Polymorphism in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. J. Bacteriol. **185:**1555–1563.
36. **Tamborrini, M., et al.** 2009. Immuno-detection of anthrose containing tetrasaccharide in the exosporium of Bacillus anthracis and Bacillus cereus strains. J. Appl. Microbiol. **106:**1618–1628.
37. **Tauveron, G., C. Slomianny, C. Henry, and C. Faille.** 2006. Variability among *Bacillus cereus* strains in the spore surface properties and influence on their ability to contaminate food surface equipment. Int. J. Food Microbiol. **110:**254–262.
38. **Thompson, B. M., and G. C. Stewart.** 2008. Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. Mol. Microbiol. **70:**421–434.
39. **Thompson, B. M., L. N. Waller, K. F. Fox, A. Fox, and G. C. Stewart.** 2007. The BclB glycoprotein of *Bacillus anthracis* is involved in exosporium integrity. J. Bacteriol. **189:**6704–6713.
40. **Todd, S. J., A. J. Moir, M. J. Johnson, and A. Moir.** 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. J. Bacteriol. **185:**3373–3378.
41. **Waller, L. N., N. Fox, K. F. Fox, A. Fox, and R. L. Price.** 2004. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. J. Microbiol. Methods **58:**23–30.
42. **Waller, L. N., et al.** 2005. Identification of a second collagen-like glycoprotein produced by *Bacillus anthracis* and demonstration of associated spore-specific sugars. J. Bacteriol. **187:**4592–4597.